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Experimental Infection of Nonenveloped DNA Virus (TTV) in *Rhesus* Monkey

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Virus fragments homologous to TTV were detected previously from an enterically transmitted outbreak of non-A–E hepatitis [Luo et al., 1999]. To test the susceptibility of the *Rhesus* monkey to this virus and to establish its transmission routes, 6 *Rhesus* monkeys were inoculated, 3 orally and another 3 intravenously. The inoculum was prepared by extracting and filtering feces collected from a patient during the incubation period identified in the described outbreak. A second group of 3 monkeys was used for the passage study. The feces and blood samples were collected for detection of the virus by polymerase chain reaction (PCR). Four animals were subjected to liver biopsies and bile aspiration by open surgery for in situ virus detection. Viremia occurred in 4–7 days after intravenous and 7–10 days after oral inoculation. The virus was excreted in feces a few days after oral infection and simultaneously with viremia after intravenous inoculation. The virus was also detected in bile during the viremic phase. There was a prolonged carrier state with persistent viremia and virus excretion in feces for more than 6 months. Serum transaminase levels were not raised during the infection. The virus was present in both the cytoplasm and nuclei of hepatocytes, but no significant pathology was found. Therefore, the *Rhesus* monkey is susceptible to TT virus infection, but the virus seems nonpathogenic. Infection of the liver may be established either by oral or parenteral inoculation. The virus may be released from liver into the blood or via bile into feces, so it may be transmitted by both blood and fecal routes. ***J. Med. Virol. 61:159–164, 2000.***

KEY WORDS: nonenveloped DNA virus (TTV); *Rhesus* monkey; experimental infection

INTRODUCTION

Over the past few decades, a bewildering array of hepatotropic viruses have been identified and are designated as hepatitis viruses A though E. A number of patients with acute or chronic hepatitis, however, do not have serum markers of infection with the known hepatitis viruses [Mast et al., 1995], indicating that some forms of viral hepatitis are caused by an unidentified hepatotropic agent(s). Recently, a novel nonenveloped DNA virus was isolated from the serum of a Japanese patient with post-transfusion hepatitis, and was named as TT virus (TTV) after the initials of the index case [Nishizawa et al., 1997; Okamoto et al., 1998a].

An outbreak of viral hepatitis occurred in a vocational school in October of 1996 and was investigated [Luo et al., 1999]. A total of 381 students were affected and the prevalence of clinical infection was as high as 60.7%. The major clinical feature of the disease was transaminase elevation with mild symptoms. The course of the disease in most patients was self-limited, but the illness was prolonged and involved relapse in some patients. Histological examination revealed mild portal hepatitis or nonspecific reactive hepatitis. Because HBsAg, anti-HBc IgM, anti-HCV and HCV RNA were not detected in the patients, hepatitis E was particularly considered. The diagnosis was excluded, however, by testing serum anti-HEV IgM by four independent laboratories. None of the patients had a history of transfusion and only a few had occasional injections. Therefore, casual contact or small-scale food transmission were considered to be risk factors for the outbreak.

To determine what pathogen caused the hepatitis-like outbreak, we examined retrospectively stored sera collected from patients in the acute phase as well as stool samples over a period of 2 weeks before onset. A viral genome fragment highly homologous to TTV was detected in the serum of 77% patients, although none had a history of transfusion. Surprisingly, the same viral fragment was also detected in the stool samples of 40% of the patients. Taken together with epidemiologi-

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cal studies, these results suggest that the virus can be transmitted enterically in addition to blood-borne routes.

To investigate whether TTV is a transmissible agent, and whether it can be transmitted orally and parenterally, an experimental infection study was carried out with *Rhesus* monkeys.

MATERIALS AND METHODS

Preparation of Fecal Extraction

Nonenveloped DNA TT virus-positive stool was collected from a patient having elevated alanine transaminase (ALT) over a period of 2 months during the outbreak described previously [Luo et al., 1999]. Stool was stored ≤−70°C. Sixteen g of feces was suspended in 70 ml of 0.01 M phosphate-buffered saline (pH 7.4) and 10 ml of chloroform. Small glass beads were added and the mixture shaken for 30 min. The stool suspension was centrifuged subsequently at 7,000 × *g* for 20 min. The resultant supernatant was passed through a 0.2 μ filter yielding a 20% (w/v) fecal extract. The 1:2 diluted extract was tested for the presence of virus via PCR.

Animal Inoculation

Ten *Rhesus* monkeys were fostered individually in separate cages for 2 months for quarantine and survey. Serum was collected biweekly to monitor ALT levels. All animals had normal serum ALT activity. Of the 7 monkeys in the first group, 3 received the fecal extract intravenously (10 ml/animal), and 3 were inoculated by irrigating stomach (20 ml/animal). The remaining monkey was administered with the virus-negative stool extract as a control. Stool and blood samples were collected daily in the first week and every week thereafter from all animals.

The passage study involved the second group of 3 monkeys, in which 2 animals were orally inoculated with virus-positive stool from a monkey in the first group and the remaining one was used as a control.

Surgical procedures were carried out on 2 orally inoculated monkeys of the first group on the 4th and 7th days after inoculation. The third procedure was carried out 6 months later. Surgical procedures involved the collection of bile and liver biopsy.

Virus Detection

DNA extraction. Using phenol-isopropanol-ethanol DNA was extracted from 50 μl of serum or 1 g of feces subsequent to centrifuging at 1,000 × *g* for 15 min to remove sediments. The DNA precipitates were resuspended in 20 μl of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Primers. Primers were designed from the previously reported TTV sequence [Okamoto et al., 1998a] as follows: the outer (nt.1714-) 5′-CAG TTA CCA ATA AAA GCA GC and (nt.2302R) 5′-TGT CTG AGT GGT ATG CAA AG, and the inner: (nt.1915-) 5′-GGC AAC ATG TTA TGG ATA GAC TGG and (nt.2185R) 5′-CTG GCA TTT TAC CAT TTC CAA AGT T, These primers resulted in the amplification of a 271-bp fragment.

Nested polymerase chain reaction (nPCR). Template DNA, primers, dNTP and Taq polymerase were mixed, and the reaction cycles were carried out as previously reported [Luo et al., 1999]. The amplified product was resolved using agarose gel electrophoresis.

Sequencing of nPCR product. The PCR products, using the inner primer, amplified from the inoculum, a stool sample of an orally administered monkey of the first group, and a stool sample of a passage monkey respectively, were directly sequenced (Bioneer Inc. St. Charles, IL.). The results were compared with the TTV sequence [Okamoto et al., 1998a] using DNASIS™ (Hitachi Software Engineering America, Ltd.).

In Situ Hybridization

Probe synthesis and labeling. The viral fragment from patient's stool sample, that was homologous to nt.1714–2302 of TTV sequence, was cloned into a pGEM-T vector (Promega Ltd.) resulting in a plasmid used as template. A probe (589 bp) was prepared via PCR using the outer primers as mentioned above, except for the substitution of dNTP with a labeling mixture (Boehringer Mannheim kit), in which 0.35 mM of 1 mM dTTP was replaced by DIG-dUTP. The probe was identified by sequencing.

Hybridization. Formalin-fixed and paraffin-embedded liver sections were dewaxed with xylene, digested with pepsin, and fixed with polymethanol. The sections were pretreated successively with 0.25% acetic anhydride for 10 min, 2× SSC 2 × 2 min, Tris-glycine for 30 min, 2× SSC 2 × 2 min, and then dehydrated with a series of concentration-increasing ethanol. Fifty μl of hybridization buffer (20× SSC 15 ml, deion formamide 22.5 ml, 50× Denhardt sol 5 ml, and dextran 5 g in H_2O 7.5 ml) containing 1 μl of probe (~50 pg) was applied on a slide. It was denatured at 95°C for 5 min, rapidly cooled on ice, and then incubated at 42°C overnight. The sections were then washed successively in 6× SSC with 45% formamide at 42°C for 2 × 10 min, 2× SSC and 0.2× SSC both at room temperature for 2 × 5 min.

Negative hybridization controls. Five reactive controls were designed as follows: (1) normal monkey liver; (2) prior digestion of the section with DNase; (3) specific probe replaced by a nonspecific HPV probe; (4) hybridization without probe present; and (5) competitive inhibition, in which non-labeling plasmid virus was mixed with the probe virus at a weight ratio of 200:1.

Detection. After hybridization and stringency washes, the viral signal of the slide was detected using a Boehringer Mannheim Kit according to manufacturer's instruction, as a color.

RESULTS

In the 3 monkeys inoculated orally with patient fecal extract, the viral fragments were present in feces on the same day as inoculation, indicating the excreted inoculum. Two monkeys stopped excreting virus on the

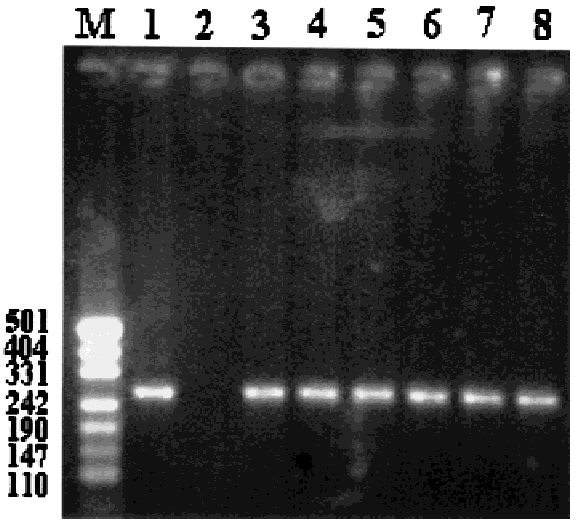

Fig.1. Detection of viral genomic fragment from monkey's stool with nPCR. M: molecular markers. **Lane 1**: positive control using a plasmid of nonenveloped DNA virus cloned with PCR product, that was identified by sequencing. **Lane 2**: feces sample from negative control monkey. **Lane 3–5**: feces from 3 orally inoculated monkeys collected at the 3rd, 4th and 4th day after inoculation respectively. **Lane 6–8**: feces from 3 intravenously inoculated monkeys collected at the 7th, 9th and 10th day after inoculation respectively. All were the 1st day of viral appearance. The expected 271 bp bands were shown.

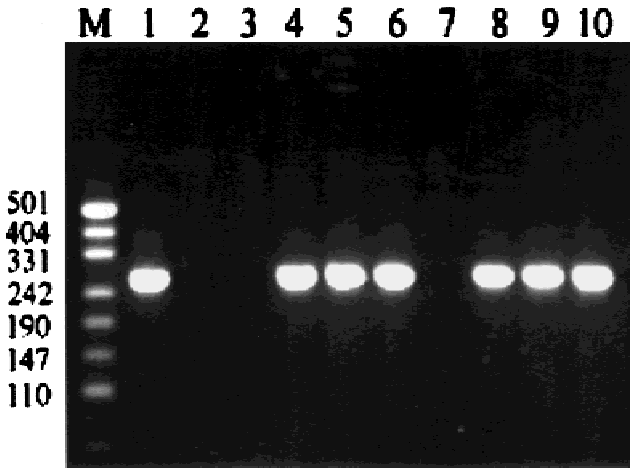

Fig. 2. Detection of viral genomic fragment from serial blood and stool samples in one of intravenously inoculated monkeys. M: molecular markers. **Lane 1**: positive control using a plasmid of nonenveloped DNA virus cloned with PCR product, that was identified by sequencing. **Lane 2**: serum sample from negative control monkey. **Lane 3–6**: the blood samples collected at the 6th, 7th, 60th and 180th day after inoculation respectively. **Lane 7–9**: the feces samples collected at the 7th, 9th, 60th and 180th day after inoculation respectively. The nPCR results showed expected 271 bp bands.

second day and re-evacuated by the 3rd or 4th days (Fig. 1). All 3 had positive feces thereafter. Viremia in these 3 animals began 7–10 days after inoculation.

The blood of the 3 monkeys that were intravenously inoculated in the first group became virus-positive 4–7 days after the inoculation. Viral fragments were also detected in these stools at about the same time (Fig. 2).

In the passage study, 2 animals were inoculated orally with the virus-positive fecal extract from one monkey of the first group. The viral fragments were also detected in feces with the same time course as seen in the first group.

The PCR products were amplified from the inoculum, the feces of a monkey inoculated orally, and the feces of a passage monkey. A 222 bp fragment, remained after abstracting the primer domains, was sequenced. Overall homology of the inoculum sequence compared with the published TTV domain sequence [Okamoto et al., 1998a] was 96.4%, that of the feces from the monkey inoculated orally was 99.6%, and that of the feces of passage monkey with the original monkey was 98.7% (Fig. 3).

The results of parallel tests of stool and blood samples from control animals were all negative.

During the 6 months of observation the viral fragments were always detected in the stool and blood samples of all inoculated animals. Serum ALT was determined every 2 weeks, to be normal during the infection course.

Six bile samples were collected from the opening of common bile duct in the duodenum by squeezing the gallbladder to prevent contamination by blood. Two virus-negative samples were collected on the 4th day after inoculation. Viremia was also not present. The remaining 4 bile samples, collected during the viremic phase, either on the 7th day or after 6 months were positive for the virus.

To follow the infection course of 2 monkeys inoculated orally, bile samples were aspirated and the liver was biopsied on the 4th and 7th days or after the 6th month after inoculation. Viral signals were detectable in the liver on the 4th day via in situ hybridization, although the viremia was not present and the bile was still virus-negative. Fecal virus at this time may be derived from the intestinal wall (unpublished data). The viremia detected from the 7th day persisted even after 6 months. During this period, the viral signals in liver increased, and the bile became virus-positive. By this time the virus fragments detected in feces may have been derived mainly from the liver.

The viral markers were present in cytoplasm, nuclei, or both of the hepatocytes, reflecting its replication and migration in the cell. The positive hepatocytes were dispersed in the lobule, and most seemed to be in peripheral of the portal (Fig. 4); however, there were no significant pathological changes in the liver.

DISCUSSION

An outbreak of enterically transmitted viral hepatitis, that is not of types A or E has been reported [Luo et al., 1999]. A viral DNA clone (accession AF 080449, GenBank) was isolated from the stools of the patient during the outbreak. Until now, 5 viral fragments from feces have been amplified (unpublished data). These fragments can be aligned with 2.8 kb sequence expanded over a region containing nucleotides 634–3431, occupying 75% of the published 7.5 kb TTV sequence [Okamoto, et al. 1998a]. There is an overall homology

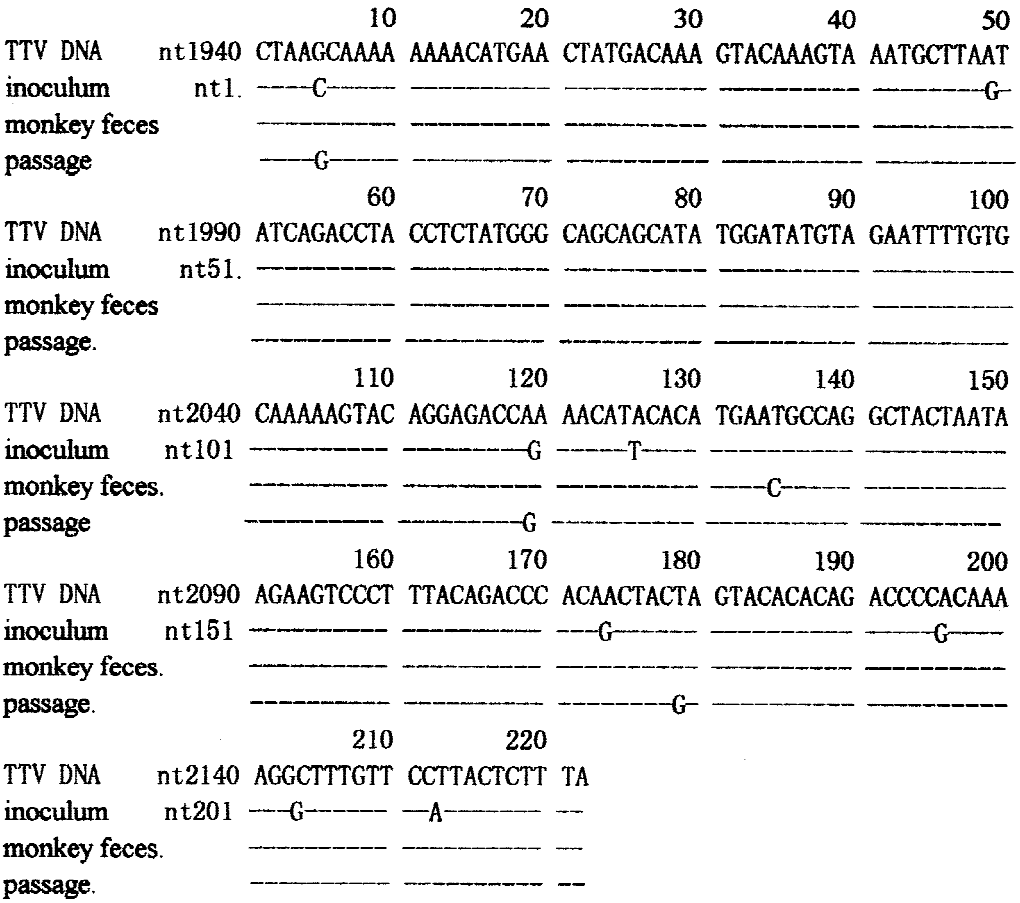

Fig. 3. Alignment of the PCR products from stool samples of a monkey orally inoculated and of a passage monkey, compared with published TTV sequence [Okamoto et al., 1998a]. The 271 bp was directly sequenced with a pair of inner primers. The 222 bp of fragment left after the terminal primer sequences were excluded. – = a nucleotide identical to sequence above.

of 97.1%. A BLAST™ search against this sequence retrieved no other sequence than TTV.

The TT virus is also thought to be a transfusion-transmitted virus [Nishizawa et al., 1997; Okamoto et al., 1998a]. Most cases reported previously involved infection by blood transmission [Nishizawa et al., 1997; Okamoto et al., 1998a; Simmonds et al., 1998; Charlton et al., 1998; Tanaka et al., 1998; Jiang et al., 1999], although some infection routes were undefined [Charlton et al., 1998; Tanaka et al., 1998; Naoumov et al., 1998; Yamamoto et al., 1998; Hohne et al., 1998].

These viral fragments have been detected in both stool and blood samples collected, however, from patients during an outbreak of fecal-orally transmitted hepatitis [Luo et al., 1999]. Recently, the fecal excretion of TT virus was demonstrated in 5 patients with post-transfusion hepatitis [Okamoto et al., 1998b]. Bile excretion was also shown in all 5 patients with TT viremia [Ukita et al., 1999]. Liver virus titer was much higher than in serum [Okamoto et al., 1998a], suggesting that TTV may replicate in liver. Accordingly, TTV would be secreted from liver into bile and then into feces enabling transmission by a fecal-oral route.

In the present study, the high homology between the inoculum and the feces of inoculated monkeys and of passage monkeys indicates that the infection was derived from its corresponding inoculum. It has been shown that the *Rhesus* monkey is susceptible to this virus. Recently, it was also shown that TTV can be transmitted to chimpanzees [Mushahwar et al., 1999]. It may be concluded therefore, that some of nonhuman primates are susceptible to the nonenveloped DNA virus, but neither *Rhesus* monkeys nor chimpanzees exhibited any biochemical or histological evidence of hepatitis.

After experimental infection of the nonenveloped DNA virus in *Rhesus* monkeys, viremia develops and the virus appears in bile and in feces after oral or intravenous inoculation. The latent period is only a few days, and the duration of viremia and enteric viral carriage can be more than 6 months. We suggest that the virus replicates in the liver, from where it enters the blood stream. It is also suggested that the virus from the liver is excreted into the bile, followed by viral shedding into the feces. This indicates both fecal-oral and parenteral transmissibility of the virus, a unique characteristic among hepatotropic viruses. The dual modes of transmission may account for the wide spread

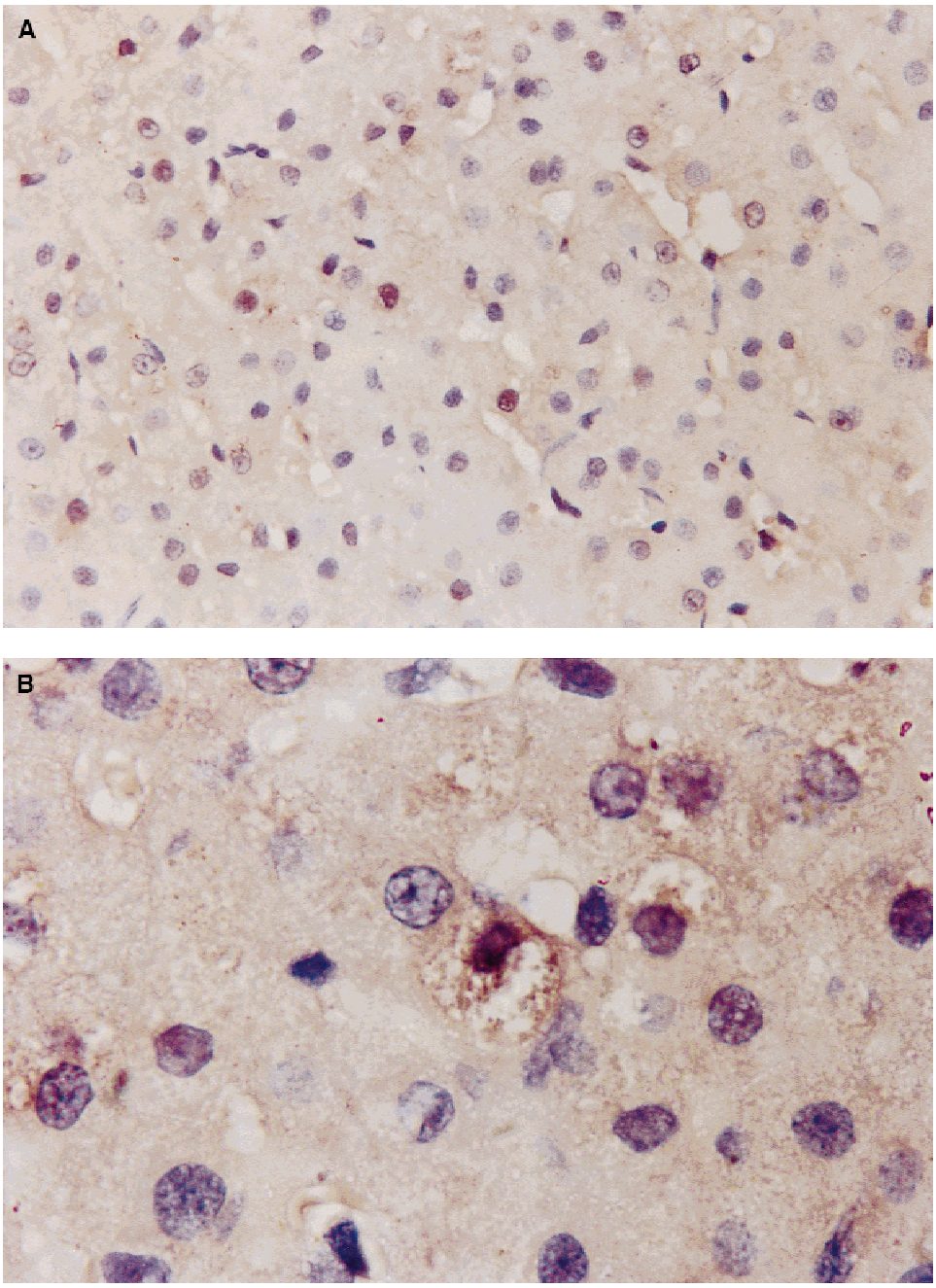

Fig. 4. In situ hybridization for the nonenveloped DNA virus in hepatocytes of experimental monkey. (**A**) and (**B**) show granular positivity for both the cytoplasm and nuclei (digoxigenin-labeled virus DNA with NBT).

infection within communities. In China, viral DNA was detected in 10.6% of, apparently healthy blood donors [Jiang et al., 1999] and in 9.1% or 10.0% of serum and stool samples, collected respectively from inhabitants of a village [Zhang et al., 1998].

According to its molecular and biophysical characteristics, the TT virus is similar to *Parvoviridae* [Okamoto et al., 1998a] or *Circoviridae* [Mushahwar et al., 1999]. The animal parvoviruses, such as feline parvovirus and mink enteritis virus, are shed into feces [Pattison, 1990]. The porcine circovirus was also isolated from fecal samples in pigs infected experimentally [Tischer et al., 1986]. TTV, however, is the first human DNA virus to present itself in feces.

The pathogenesis of the nonenveloped DNA virus is questionable. In this study, a viral marker was detected in monkey hepatocytes using in situ hybridization. In the outbreak described previously, the virus was able to induce an elevation of transaminase levels with mild lobular hepatitis and portal infiltration [Luo

et al., 1999]. It is reasonable to postulate that TTV is a weak pathogenic virus for the liver in some individuals. Further, in this study the monkeys carried the virus for more than 6 months and in follow-up studies, ALT elevation was 29.6% and 13.9%, whereas viral detection in blood was 68.5% and 18.5% at 6 and 18 months, respectively [author's unpublished data]. These results imply that both liver damage and viremia can become chronic.

The results of animal experiments in this study and the observations in the outbreak reported previously [Luo et al., 1999], confirmed that the TT virus might be transmitted by both blood and enteric routes, and might induce both acute and chronic infection.

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